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(54) Title: RECOMBINANT PROTEIN PRODUCTION IN URINE

(57) Abstract

The present invention provides methods which generate a polypeptide that is secreted into the urine by using the uromodulin gene promoter, or other promoters from genes whose products are specifically expressed in the kidney, to direct the expression of the secreted polypeptide. Also disclosed is a transgenic animal secreting a recombinant polypeptide into its urine, as well as a method for detecting such an animal while the animal is *in utero*. Also disclosed are methods for generating a polypeptide that is secreted into the conditioned media of cultured kidney cells transformed with a polypeptide–encoding nucleic acid sequence that is operably linked to a uromodulin gene promoter, or other kidney specific gene promoter.

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RECOMBINANT PROTEIN PRODUCTION IN URINE

Background of the Invention

The invention relates to transgenic animals capable of secreting recombinant polypeptides in their urine.

The generation of recombinant proteins utilizing an animal as a bioreactor has the advantage of producing a recombinant protein that is likely properly folded. In addition, since animals can reproduce, they provide an almost inexhaustible source of the recombinant protein. Production of recombinant proteins in animal fluids has been used widely. Examples include the secretions of recombinant proteins in milk using milk specific promoters.

Urine presents an advantage over the milk specific expression of recombinant polypeptides for the following reasons: (1) the process of recombinant polypeptide production from urine is initiated immediately after birth (or even prior to birth); (2) unlike the lactation process, urine-specific recombinant polypeptide production doesn't depend on a hormonal or reproductive status of the transgenic animal; and (3) both female and male animals can be used for recombinant polypeptide production from urine. In addition, urine naturally contains very small amounts of proteins as compared to milk, thus facilitating the isolation of recombinant polypeptides from the urine of transgenic animals.

It has recently been reported than recombinant proteins can be secreted in urine using the bladder specific promoter of the uroplakin II gene. Specifically, small amounts of recombinant human growth hormone (rc hGH) were secreted in the urine of transgenic mice (150 μg/liter) under the influence of the uroplakin II promoter (Kerr et al., Nature Biotechnology 16: 75-79, 1998). However, since the bladder is not a secretory organ, the secretion of recombinant proteins using the uroplakin II promoters may be hampered.

Summary of the Invention

The present invention provides a transgene that includes a desired polypeptide whose expression is driven by the uromodulin gene promoter, or another promoter from a gene whose product is specifically expressed in the kidney, thus allowing the expression and secretion of the polypeptide from the kidney into the urine, from which it can be isolated.

Accordingly, in a first aspect, the invention provides transgenic non-human urine secreting animals which are capable of producing recombinant polypeptide that are secreted extracellularly into the urine by the kidney tissue of the animal. The animals may be mammals, and may be ruminants, or non-ruminants. Representatives of non-human urine-secreting mammals useful in the invention include, without limitation, rodents, rabbits, pigs, goats, sheep, horses, and cows.

In one embodiment of the first aspect of the invention, the recombinant polypeptide is an enzyme that is able to degrade or catalyze a degradation reaction of undesirable components of urine (*e.g.*, ammonia).

In a second aspect, the invention provides a method for obtaining urine of a transgenic animal that includes the steps of: (a) generating a transgenic construct composed of the controlling elements of a kidney specific gene (including the 5'-end promoter sequences and 3'-end elements) operably linked to the nucleic acid sequence of interest to be expressed; (b) screening the construct before a transgenic animal is generated; this construct screening could be done in kidney epithelial lines; (c) inserting into the genome of a non human animal the transgenic construct; (d) collecting the urine from this non-human animal; and (e) isolating the product from the animal's urine. Preferably, the kidney specific gene is the uromodulin gene.

In a third aspect, the invention features a transgene useful for the generation of a transgenic animal, where transgene includes: (a) a promoter

from a kidney specific gene that is functional in the kidney secretory cells of the transgenic animal of interest, (b) a leader sequence that is functional in the kidney secretory cells of said transgenic species and (c) a nucleic acid sequence encoding a recombinant endogenous or exogenous product. Preferably, the kidney specific gene is a uromodulin gene. Preferably, the leader sequence is operably linked to the nucleic acid sequence to form a functional transgene that is capable of directing the expression of the secreted recombinant polypeptide (encoded by the nucleic acid sequence) in kidney secretory cells of the transgenic animal. Preferably, the kidney specific gene promoter is from the same species of animal as the of the transgenic animal (*e.g.*, a promoter from a goat uromodulin gene is used to generate a transgenic goat).

In one embodiment of the third aspect of the invention, the transgene also contains in the 5' or 3' region at least one copy of insulator element sequence or a matrix attachment region. Preferably, the transgene includes four functional regions: (i) the insulator element sequence; (ii) a kidney specific expression regulation portion (*e.g.*, the uromodulin gene promoter); (iii) a leader sequence; and (iv) a nucleic acid molecule encoding a polypeptide of interest. The nucleic acid sequence encoding the polypeptide of interest may be cDNA or genomic DNA, or may encode more than one polypeptide, or a hybrid of two different proteins with domains including two different activities (*e.g.*, hybrid polypeptide containing the Fc portion of an immunoglobulin fused to insulin).

In a fourth aspect, the invention provides urine from a transgenic non-human mammal, where the urine is characterized by containing an endogenous or exogenous recombinant polypeptide, and is secreted by a transgenic animal. The transgenic animal is produced by introducing into its genome a transgene containing a nucleic acid sequence encoding the recombinant polypeptide of interest, where the nucleic acid sequence is operably linked to controlling

elements from a kidney specific gene (*e.g.*, the uromodulin gene). In one embodiment, the controlling elements are a gene promoter.

In a fifth aspect, the invention provides a nucleic acid molecule including (i) a nucleic acid sequence encoding a polypeptide, (ii) a promoter from a kidney specific gene (*e.g.*, the uromodulin gene), where the promoter is operably linked to the sequence, and where the promoter is not naturally associated with the nucleic acid sequence, and (iii) a leader sequence that enables secretion of the polypeptide by the urine-producing cells into urine of an animal. In various embodiments, the kidney-specific gene is selected from the group consisting of a cow, a human, and a rodent, and the animal is a mammal or is selected from the group consisting of a rodent and a ruminant (*e.g.*, a cow, sheep, or goat). The polypeptide may have biological activity or may be soluble.

In a sixth aspect, the invention provides an animal in which the genome of cells that contribute to urine production in the animal includes a nucleic acid molecule including (i) a nucleic acid sequence encoding a polypeptide, (ii) a promoter from a kidney specific gene (*e.g.*, a uromodulin gene), where the promoter is operably linked to the sequence, and where the promoter is not naturally associated with the nucleic acid sequence, and (iii) a leader sequence that enables secretion of the polypeptide by the urine-producing cells into urine of an animal. In various embodiments, the cells are kidney secretory cells, the animal is a selected from the group consisting of a rodent and a ruminant (*e.g.*, a cow, sheep, or goat), and the animal is a mammal.

In a seventh aspect, the invention features a method for producing a polypeptide that is secreted in the urine of an animal, the method including the steps of: (a) providing an embryonal cell transfected with a polypeptide-encoding nucleic acid molecule operably linked to a kidney specific gene promoter (*e.g.*, a uromodulin gene promoter) that expresses and causes

secretion of the polypeptide from a kidney cell derived from the transfected embryonal cell, where the promoter is not naturally associated with the nucleic acid sequence; (b) growing the embryonal cell to produce an animal including polypeptide expressing and secreting cells; and (c) isolating the protein from the polypeptide expressing and secreting cells of the animal. Preferably, the animal is a mammal.

In an eighth embodiment, the invention features a method for producing a polypeptide, the method including the steps of: (a) providing a cell of an animal, the cell transfected with a nucleic acid molecule that contains (i) a nucleic acid sequence encoding a polypeptide, (ii) a kidney specific gene promoter (*e.g.*, a uromodulin gene promoter) that directs expression of the polypeptide in the cell, where the promoter is not naturally associated with the nucleic acid sequence, and (iii) a leader sequence that causes secretion of the polypeptide by the cell; (b) culturing the transfected cell; and (c) isolating the polypeptide from the culture medium of the cultured transfected cell. In various embodiments, the cell is a kidney secretory cell or is an immortalized cell. Preferably, the animal is a mammal.

In a ninth aspect, the invention features a method for producing, in the urine of a vertebrate, a recombinant protein that contains two or more subunits linked to each other by disulfide bonds; the method includes the step of: (a) providing a transgenic vertebrate exhibiting urine-specific production of the recombinant protein; (b) collecting urine from the vertebrate; and (c) isolating the protein from the urine. A related aspect of the invention is the vertebrate animal used in this method. The method can employ a uroplakin I, uroplakin II, or uromodulin promoter. In one preferred embodiment of the method, the protein, when it is excreted into the urine for the animal, is folded such that it is rendered biologically active (i.e., exhibits at least some of the biological activty of the native form of the protein). Alternatively, in order to protect the animal

from possible dilaterious effects of the active protein, the protein can be intentionally engineered to render it partially or wholly inactive at the time of secretion, but activatable by simple means following collection of the urine from the animal. Such methods are known and are described e.g., in U.S. Serial No. 08/775,842, commonly assigned with the present application.

Examples of multimeric proteins that can be produced according to this ninth aspect of the invention are monoclonal antibodies, of any isotype; and heterodimeric fertility hormones. The main hormones in this category are follicle stimulating hormone (FSH), lutenizing hormone (LH), and human chorionic gonadotrophin (hCG). Each of these hormones is a glycoprotein containing an alpha and a beta subunit; the alpha subunit of all three is identical, while the beta-subunits differ and confer specificity of biological action on each hormone. FSH and LH are important commercial products which have been purified from human urine. These hormones currently are made in recombinant form in cultured mammalian cells. The sequences of all of these hormones are known. For example, PCT Application WO 90/02757 gives the sequences of LH and FSH.

The process of the ninth aspect of the invention can also be used to produce another important commercial hormone product, pregnant mare serum gonadotrophin (PMSG), which is a heterodimeric glycoprotein containing an alpha and a beta subunit. The method can also be used to make inhibins and activins, which are also heterodimeric glycoproteins that are produced in the gonads. Mature inhibin consists of an αC-subunit with either a βA-or a βB-subunit. Members of this family of dimers include inhibin A, inhibin B, activin A, activin AB, and activin B (which is a homodimer, a class of multimeric proteins also included in the invention).

The process of the ninth aspect of the invention can also be used to make any of the multiple forms of collagen, including homotrimeric and

heterotrimeric forms; the sequences of collagen chains are known, and disclosed, e.g., in PCT Application WO 96/03051. The method for the ninth aspect of the invention can also be used to produce fibrinogen, which is a heterotrimeric protein whose sequences are known, e.g., PCT Application WO 95/22249.

The invention also provides multiple transgenes encoding various polypeptides or versions of the same polypeptide (*e.g.*, a polypeptide containing conservative amino acid substitutions, or amino acid substitutions that would enhance the stability of the polypeptide). These multiple transgenes may be coinjected (*i.e.*, co-microinjected) simultaneously, or sequentially. Thus, two or more recombinant polypeptides are secreted in the transgenic animal's urine.

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation).

By "naturally associated" is meant that two sequences (*e.g.*, a promoter and a polypeptide-encoding sequence) are operably linked in the naturally occurring genome of the organism from which the two sequences are derived. For example, the bovine uromodulin gene promoter is naturally associated with the bovine uromodulin-encoding sequence .

By "not naturally associated" is meant that two sequences (*e.g.*, a promoter and a polypeptide-encoding sequence) are not operably linked in the naturally occurring genome of the organism from which one or both of the two sequences are derived. For example, the goat uromodulin gene promoter is not naturally associated with the bovine uromodulin-encoding sequence. In addition, the goat uromodulin gene promoter is not naturally associated with the human tPA-encoding sequence.

By "kidney specific gene" is meant a gene whose product is expressed

only in kidney cells. One preferred example of a kidney specific gene is the uromodulin gene. Specifically excluded from the definition is are the uroplakin genes, since their products are expressed in the bladder.

By an "insulator element sequence" is meant a nucleic acid sequence which, when operably linked to a regulatory element (*e.g.*, a promoter) directing the expression of a nucleic acid molecule of interest on a transgene, allows for the expression of the nucleic acid molecule, regardless of the position of the genome in which the transgene has integrated. Typically, an insulator sequence is located immediately 5' to a promoter sequence.

By a "leader sequence" or a "signal sequence" is meant a nucleic acid sequence which, when operably linked to a nucleic acid molecule of interest, allows for the secretion of the product of the nucleic acid molecule. The leader sequence is preferably located 5' to the nucleic acid molecule. Preferably, the leader sequence is obtained either from same gene as the promoter that is used to direct the transcription of the nucleic acid molecule, or is obtained from the gene from which the nucleic acid molecule of interest is derived.

By a "transfected cell" or a "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant molecular biology techniques, a nucleic acid molecule encoding a polypeptide of the invention. Preferably, the cell is a eukaryotic cell from a multicellular animal (*e.g.*, a mammal).

By an "embryonal cell" is meant a cell that is capable of being a progenitor to all the somatic and germ-line cells of an organism. Exemplary embryonal cells are embryonic stem cells (ES cells) and fertilized oocytes. Preferably, the embryonal cells of the invention are mammalian embryonal cells.

By "germ-line cell" is meant a eukaryotic cell, progenitor, or progeny thereof, which is a product of a meiotic cell division.

By "operably linked" is meant that a nucleic acid sequence and one or more regulatory sequences (*e.g.*, a promoter) are connected in such a way as to permit expression and/or secretion of the product (*i.e.*, a polypeptide) encoded by the nucleic acid sequence when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequences.

By "endogenous," as used herein in reference to a gene or a polypeptide, is meant a gene or polypeptide that is normally present in an animal.

By "exogenous," as used herein in reference to a gene or a polypeptide, is meant a gene or polypeptide that is not normally present in an animal. For example, human growth hormone is exogenous to a transgenic goat.

By "transgene" is meant any piece of nucleic acid that is inserted by artifice into a cell, or an ancestor thereof, and becomes part of the genome of the animal which develops from that cell. Such a transgene may include a gene which is partly or entirely exogenous (*i.e.*, foreign) to the transgenic animal, or may represent a gene having identity to an endogenous gene of the animal.

By "transgenic" is meant any cell which includes a nucleic acid sequence that has been inserted by artifice into a cell, or an ancestor thereof, and becomes part of the genome of the animal which develops from that cell. Preferably, the transgenic animals are transgenic mammals (*e.g.*, rodents or ruminants). Preferably the nucleic acid (transgene) is inserted by artifice into the nuclear genome.

By "reporter gene" is meant any gene or nucleic acid molecule which encodes a product whose expression is detectable. A reporter gene product may have one of the following attributes, without restriction: fluorescence (*e.g.*, green fluorescent protein), enzymatic activity (*e.g.*, luciferase or chloramphenicol acetyl transferase), toxicity (*e.g.*, ricin), an ability to confer resistance to a reagent (*e.g.*, resistance to neomycin by the neo gene or resistance to copper by the metallothionein-encoding gene), an ability to confer

susceptibility to a reagent (*e.g.*, susceptibility to gancyclovir by the Herpes Simplex Virus thymidine kinase-encoding gene), or an ability to be specifically bound by a second molecule, such as biotin or a detectably labelled antibody (*e.g.*, binding by biotin by the avidin-encoding gene or binding by an antibody (*e.g.*, detectably labelled) by a cell surface expressed epitope-encoding gene).

Brief Description of the Drawings

Fig. 1 shows the partial DNA sequence of the human uromodulin gene promoter (GenBank Accession No. S75968; Yu et al., Gene Expr. 4: 63-75, 1994).

Fig. 2 shows the partial DNA sequence of the bovine uromodulin gene promoter (GenBank Accession No. S75961; Yu et al., *supra*).

Fig. 3 shows the partial DNA sequence of the rat uromodulin gene promoter (GenBank Accession No. S75965; Yu et al., *supra*).

Fig. 4 is a schematic diagram illustrating an example of a method to generate a uromodulin promoter transgenic construct. The nucleic acid sequence (flanked by BamHI and SpeI sites) in this figure may encode human tPA (see Fig. 5), or may encode a reporter gene, such as luciferase.

Fig. 5 is a schematic diagram of a PCR reaction to generate a human tPA-encoding cDNA fragment flanked by a BamHI recognition sequence on the 5' end and a SpeI recognition sequence on the 3' end.

Fig. 6 is a schematic diagram of a transgenic construct that includes a goat uromodulin gene promoter directing the expression of a human tPA-encoding sequence. The backbone plasmid has a hygromycin resistance gene for eukaryotic cell selection, an ampicillin resistance gene for prokaryotic cell selection, and a ColE1 origin of replication for amplification in bacteria.

Figs. 7-9 are schematic representations of the construction of expression vectors of the invention.

Fig. 10 is the sequence of the goat UM promoter.

Detailed Description

The present invention relates to a process for excreting recombinant proteins in the urine of non-human animals. This process uses expression vectors containing promoter sequences based on the regulatory elements of uromodulins (also called the Tamm-Horsfall glyoprotein (THS) / uromucoid), or the promoter sequencess from other kidney-specific genes to express recombinant proteins in the kidney, thus allowing their secretion into urine.

Uromodulin is synthesized by the kidney and localizes in the early distal tubule and the renal ascending limb. Uromodulin is the most abundant protein in human (30-45 mg/24 hours) and rat urine (0.5-2.9 mg/24 hours) (Gokhale et al., Urol. Res. 25: 347-354, 1997). No uromodulin protein has been detecting in normal tissues other than the kidney (Howie, A.J., J. Pathol. 153: 399-404, 1987), however cross-reacting proteins with antibodies against uromodulin have been identified in human and rat sera at low-levels (Lynn and Marshall, Biochem. J. 194: 561-568, 1981; Wirdnam and Milner, Nephron 40: 362-367,1985). Antibodies raised against uromodulin crossreact with the loop of Henle in the kidney of mammals, superficial layers of the skin of several amphibians and fish, superficial layers of the oral mucosa and gills of fish, and the distal tubules of the kidney of amphibians. No cross reaction is observed in avian and reptile species (Howie et al., Cell Tissue Res. 274: 15-19, 1993).

Uromodulin is a 616-amino acid, 85 kDA glycoprotein with *in vitro* immuno-suppressive properties. The partial bovine and rodent uromodulin promoters have been cloned and shown to contain the typical controlling transcriptional elements in the proximal promoter (Yu et al., Gene Expr. 4: 63-75, 1994). Using the uromodulin promoter is useful for generating urine-secreted proteins because, since the level of uromodulin can be increased by

increasing the urine volume, a means is thus provided for increasing the total output of the recombinant product, whose expression is directed by the uromodulin gene promoter. In addition, since uromodulin is secreted in the urine of the fetus, amniotic fluid sampling may allow early detection of a transgenic fetus expressing a recombinant (rc) polypeptide whose expression is directed by the uromodulin gene promoter (see the procedure of Phimister and Marshall, Clin. Chim. Acta 128: 261-269, 1983).

The Transgenic Construct

For the generation of a transgenic construct that allows for the secretion of a recombinant polypeptide from kidney secretory cells, any appropriate backbone may be used. Where the nucleic acid sequence encoding the recombinant polypeptide of interest is genomic DNA, the backbone plasmid may be derived from a cosmid (*e.g.*, SuperCos or pWE15, both commercially available from Stratagene, La Jolla, CA).

Preferably, the backbone has a prokaryotic origin of replication, as well as a gene encoding a selectable marker that may be used for prokaryotic cells (*e.g.*, ampicillin, tetracycline, and chloramphenicol), for easy propagation and amplification in transformed bacteria. Prior to microinjection, the complete transgenic construct may be linearized by removing all the bacterial sequences (*i.e.*, the bacterial origin of replication and the bacterial selectable marker gene).

In addition, the backbone plasmid should have a selectable marker gene that may be used for selection in a eukaryotic cell (for example, hygromycin, neomycin, puromycin, and zeomycin). Such a marker gene may be under the expression of its endogenous promoter (*e.g.*, the puromycin-resistance gene promoter directing the expression of the puromycin-resistance gene). Alternatively, a relatively weak promoter (*e.g.*, the SV40 early promoter) may

be used to direct the expression of a selectable marker gene product.

Representative polypeptides encoded by nucleic acid sequences to be expressed and secreted in the urine include, without limitation, erythropoietin (EPO), human tissue plasminogen activator (htPA), insulin, antibodies (*e.g.*, monoclonal or humanized), and hormones (*e.g.*, human growth hormone).

The basic transgenic construct contemplated includes the following elements: the plasmid backbone; a kidney specific gene promoter (*e.g.*, the uromodulin gene promoter) operably linked to a leader sequence and a nucleic acid sequence encoding a polypeptide of interest; and a polyadenylation signal located 3' to the stop codon of the nucleic acid sequence. The leader sequence may be derived either from the gene whose promoter is being employed, from the nucleic acid sequence, or from an alternate secreted protein-encoding sequence (*e.g.*, the Igκ gene). Likewise, the 3' UTR, which includes the polyadenylation signal, may be from the gene from which the promoter is derived, from the nucleic acid sequence of interest, or from an alternate source (*e.g.*, the SV40 virus). A 5' UTR may be located between the promoter sequence and the leader sequence, and may be from the gene whose promoter is being employed, the nucleic acid sequence of interest, or from an alternate source. For example, where no convenient restriction enzyme recognition sequence exists in a nucleic acid sequence of interest between the 5' UTR, the leader sequence, the nucleic acid sequence (*i.e.*, the coding sequence), and the 3' UTR (which includes the polyadenylation signal), the transgenic construct may be generated in a three part ligation of the linearized backbone plasmid, the kidney specific gene promoter sequence (flanked by appropriate linkers), and the following fragment, likewise flanked by appropriate linkers: 5' UTR, leader sequence, nucleic acid sequence encoding the polypeptide of interest, and 3' UTR.

The Uromodulin Gene Promoter, an Exemplary Kidney-Specific Gene Promoter

The partial sequences of human, bovine and rat uromodulin gene promoter have been described (Figs. 1-3, Yu et al., *supra*). In addition, the GenBank sequence database provides a number of uromodulin sequences from a variety of mammals, including human (Accession Nos. M15881 and M17778). Using standard molecular biology techniques, the sequence of the uromodulin promoter from a particular animal (*e.g.*, a goat) may be isolated from genomic DNA from that animal using standard library screening techniques (see techniques in, *e.g.*, Ausubel et al., *supra*).

In addition, since only part of the promoter sequence has been elucidated, the remainder of the promoter may be derived using standard primer extension protocols or a PCR-based "gene walking" technique (using, for example, the GenomeWalker™ kits commercially available from Clontech Laboratories, Inc., Palo Alto, CA).

Once the uromodulin gene promoter is identified, it can be operably linked to a reporter sequence, such as a sequence encoding luciferase. This reporter construct may be used to test the ability of the cloned promoter to express and secrete luciferase from transformed cells. For example, following transformation of kidney cells (*e.g.*, COS cells) with a construct of the uromodulin gene promoter operably linked to the luciferase encoding sequence, the culture media of the cells may be quickly assayed for the presence of luciferase (using, for example, the luciferase detection assay kit commercially available from Promega Corp., Madison, WI).

Polypeptide Purification

Once the recombinant protein is expressed in the urine, it can be purified using standard protein purification techniques, such as affinity chromatography

(see, *e.g.*, Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994). In an example where the recombinant protein is human epidermal growth factor (EGF), the urine may be added to an affinity column to which are immobilized anti-human EGF antibodies (commercially available from, for example, Upstate Biotech. Inc., Lake Placid, NY). Once isolated, the recombinant protein can, if desired, be further purified by *e.g.*, by high performance liquid chromatography (HPLC; *e.g.*, see Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, eds. Work and Burdon, Elsevier, 1980).

Generation of a Transgenic Animal

Transgenic constructs are usually introduced into cells by microinjection (Ogata *et al.*, U.S.P.N. 4,873,292). A microinjected zygote is then transferred to an appropriate female resulting in the birth of a transgenic or chimeric animal, depending upon the stage of development of the zygote when the transgene integrated. Chimeric animals can be bred to form true germline transgenic animals.

In some methods of trangenesis, transgenes are introduced into the pronuclei of fertilized oocytes. For some animals, such as mice, fertilization is performed *in vivo* and fertilized ova are surgically removed. In other animals, the ova can be removed from live, or from newly-dead (*e.g.*, slaughterhouse) animals and fertilized *in vitro*.

Alternatively, transgenes can be introduced into embryonic stem cells (ES cells). Transgenes can be introduced into such cells by electroporations, microinjection, or any other techniques used for the transfection of cells which are known to the skilled artisan. Transformed cells are combined with blastocysts from the animal from which they originate. The cells colonize the embryo, and in some embryos these cells form the germline of the resulting

chimeric animal (Jaenisch, R., Science 240: 1468-1474, 1988). Alternatively, ES cells can be used as a source of nuclei for transplantation into an enucleated fertilized oocyte, thus giving rise to a transgenic animal.

Multiple Transgenes

In accordance with the production of recombinant protein in the urine of a transgenic animal, where the protein is composed of two different subunits non- covalently bonded to one another, it may be desirable to produce both subunits from the same transgenic animal. In such a situation, two different transgenic constructs, each encoding one of the two subunits, may be simultaneously or sequentially co-microinjected into the same zygote to produce a transgenic animal expressing both subunits.

Alternatively, nucleic acid encoding each of the two subunits can be cloned into the same expression cassette with the insertion of an intervening ribosomal entry site (IRES) (Jang *et al.*, J. Virol. 62: 2636-2643, 1988; Gurtu et al., Biochem. Biophys. Res. Comm. 229: 295-298, 1996). The advantage to cloning both nucleic acid sequences into the same expression cassette is that only one single construct is needed to generate the transgenic animal.

It will be understood that the procedures described above may be also used to generate a transgenic animal secreting two full length, unrelated proteins (*e.g.*, a recombinant insulin polypeptide and a recombinant human tissue plasminogen activator polypeptide).

Pre-Injection Screening

Prior to the microinjection of a transgenic construct encoding a desired polypeptide, the construct may be screened in cultured kidney cells *in vitro* for an ability to encode a polypeptide that is expressed and secreted by the transfected cultured cells. Cultured kidney epithelial cells, such as COS cells

or MDCK cells (both commercially available from the American Type Culture Collection (ATCC, Rockville, MD), may be transformed with the transgenic construct using any standard transformation protocol (*e.g.*, $CaPO_4$ precipitation, DEAE-dextran, electroporation; see Ausubel et al., *supra*). Since a the kidney specific gene promoter (*e.g.*, the uromodulin promoter) is active in these cells, the desired polypeptide encoded by the transgenic construct will be expressed and secreted by the transformed cells if the transgenic construct is functional. The conditioned culture media of the transformed cells may then be assayed for the presence of the secreted recombinant polypeptide.

In Utero Screening

Smaller mammals (*e.g.*, rodents) have a reasonably short gestation period, thus allowing a rapid determination of whether or not the transgenic animal is truly transgenic and able to produce the recombinant protein in its urine. However, for larger animals (*e.g.*, cattle), it may be desirable to determine whether or not the fetus is indeed transgenic and capable of producing the recombinant protein in its urine prior to birth.

Fetal renal function starts early during gestation and uromodulin is detectable in the amniotic fluid, implying, of course, that the uromodulin promoter is active. Hence, since the fetus secretes urine into the amniotic fluid of its placenta, amniotic fluid may be removed and tested for the presence of the recombinant polypeptide whose expression is directed by a uromodulin promoter. Such testing may be by any standard immmunological assay (*e.g.*, ELISA, Western blotting analysis), or, if no specific antibodies are available, by purification of the recombinant polypeptide and N-terminal sequencing.

The following examples are to illustrate the invention, and are not meant to limit the invention in any way. In addition, although the following examples describe the uromodulin gene promoter from the goat, it will be understood that

a uromodulin gene promoter from another species is also contemplated by the invention. Furthermore, other kidney specific gene promoters, whether they direct the expression of an intracellular or secreted protein, are also within the invention. Where a kidney specific gene promoter that directs the expression of a non-secreted protein is used, the leader sequence may be from the uromodulin gene, the nucleic acid sequence encoding the desired polypeptide, or any other secreted polypeptide.

Example I

The uromodulin promoter sequence may be cloned using standard techniques (*e.g.*, hybridization under non-stringent conditions) to isolate a uromodulin promoter sequence using as a probe one of the known partial uromodulin promoter sequences (*i.e.*, the rat, human, or bovine sequence). Of course, the animal desired to be made transgenic will affect which of the known partial sequences will be used as a probe. For example, should a transgenic goat be desired, the partial sequence of the bovine uromodulin promoter (provided in Fig. 2; Yu et al., *supra*) may be radiolabelled and used to probe genomic DNA prepared from goat tissues (generated according to standard techniques from goat cells). Should goat uromodulin promoter thus isolated turn out to be less than the full length promoter, the full length promoter may be isolated by extending the isolated fragment using primer extension. Alternatively, the full length promoter may be obtained using the commercially available gene walked kit commercially available from Clontech.

Once the goat uromodulin promoter sequence has been cloned, commercially available linkers (commercially available, for example, from New England Biolabs, Beverly, MA) may be attached to the ends of the promoter sequence and ligated into a bacterial plasmid containing a bacterial origin of replication (*e.g.*, the puc19 vector) for rapid amplification of the

promoter in vector-transformed *E. coli*. Thus amplified, the promoter may be freed from the puc19 vector by digestion with the restriction endonuclease which specifically cleaves at the linker sequence, and be subcloned into the transgenic construct.

Alternatively, the digested fragment can be combined with digested fragments corresponding to the leader sequence and the nucleic acid encoding the desired polypeptide (*e.g.*, tPA), and used in a four-part ligation with a EcoRI/SpeI linearized eukaryotic expression vector, a fragment containing the nucleic acid sequence of interest, and a fragment containing the 5' UTR and leader sequence (see Fig. 4). It will be understood that in the scenario of Fig. 4, a four part ligation need be used only if the 5' UTR and leader sequence are not from the uromodulin gene or the human tPA gene. Note that the backbone plasmid in Fig. 4 already contains a 3' UTR and polyadenylation signal 3' to the stop codon of the inserted human tPA-encoding nucleic acid sequence.

Example II

If a transgenic goat is desired to produce human tissue plasminogen activator (tPA), the coding sequence of the tPA gene may be generated using the known human tPA cDNA sequence (GenBank Accession No. E02027). As depicted in Fig. 5, a forward primer containing a BamHI restriction enzyme recognition site at its 5' end and a reverse primer containing a SpeI restriction enzyme recognition site at its 5' end may be used to PCR amplify a human tPA-encoding cDNA sequence from a human cDNA library (commercially available from, for example, Clontech, Palo Alto, CA). Following amplification, the PCR product may be digested with BamHI and SpeI and, as above, ligated with the fragments corresponding to the uromodulin promoter and the leader sequence with the EcoRI and SpeI linearized vector (see Fig. 4).

Example III

Prior to injecting goat zygotes with the transgenic construct shown on Fig. 4, the ability of the construct to enable a transformed cell to express and secrete a polypeptide encoded by the construction may first be determined using transformed cultured kidney epithelial cells. In this example, green monkey kidney cells (COS cells) are transformed with the construc using the $CaPO_4$ transformation method. If desired, the construct may first be linearized using a unique restriction endonuclease recognition site located within the bacterial origin of replication or the prokaryotic selectable marker gene.

Twenty-four hours following transformation, the cells' spent conditioned media is exchanged with fresh media, and the cells are returned to culture. The next day (*i.e.*, 48 hours following transfection), the conditioned media of the cells is removed and assayed for the presence of human tPA using Western blotting analysis with a human tPA-specific antibody as a probe. If desirable quantities of tPA are produced by the transformed cells, the construct may be used, as is, to microinject goat zygotes. In the alternative, where tPA is produced by the transformed cells, but in a quantity that is less than desirable, the construct may be modified, and retested in COS cells.

Example IV

After repeated screening in COS cells, a transgenic construct, such as that shown schematically in Fig. 6, may be generated. The construct shown has an insulator element sequence located upstream of the goat uromodulin promoter sequence to allow expression of the construct regardless of the site of integration. In addition, the SV40 3' UTR (which includes the SV40 polyA signal) of Fig. 4 is replaced with the 3' UTR of the goat uromodulin gene. The construct may now be linearized, if desired, by digesting the construct with XhoI and XbaI to remove the ampicillin resistance gene and the ColE1 origin

of replication (*i.e.*, the digestion fragment that includes the ColE1 origin and the ampicillin resistance gene is discarded). The remaining fragment (*i.e.*, the transgene) is next used to microinject goat zygotes.

It will be understood that in the schematic diagrams of Figs. 4-6, the particular restriction endonucleases exemplary; any suitable restriction endonuclease may be employed. In particular, where a restriction endonuclease is desired to be unique (*e.g.*, to facilitate cloning and subcloning of the goat uromodulin promoter), the linkers used to flank the sequence may be recognition sites of rare-cutting enzymes (*e.g.*, SseI or NotI).

Example V

The construct depicted in Fig. 6 can be used to generate transgenic mice capable of secreting human tPA into their urine. The generation of transgenic mice prior to the generation of transgenic goats is preferable not only because of the greater time expenditure (*i.e.*, longer gestation period) required to generate a transgenic goat as opposed to a transgenic mouse, but also because of the higher expense in maintaining and housing the animals.

Hence, mature female mice are superovulated and mated with males to produce fertilized eggs. The eggs are harvested for pronuclear microinjection. For example, a Leitz micro-manipulator and a Nikon inverted microscope may be employed for the microinjections. Pseudopregnant female mice are then implanted with microinjected two-cell embryos. Once the pups are born, their urine is screened for the presence of human tPA.

Example VI

Expression of the monoclonal antibody K20 can be achieved under the control of the mouse uroplakin II gene promoter in a eukaryotic expression vector, pcDNA4/HisMax.

K20 is a mouse monoclonal antibody ("mAb") that recognizes a particular epitope on human CD29. A soluble form of K20 was shown to block peripheral T cell activation and proliferation induced by an anti-CD3 antibody. This negative effect might be mediated by an increase in cAMP levels or an inhibition of diacylglycerol and PA formation. The *in vitro* functional effects of K20 make it a good candidate for therapeutic immunosuppression. A humanized K20 mAb (Hu-K20) has been produced with potentially reduced immunogenicity and functional properties identical with the murine mAb K20 (Paul, M.A. et al., *Mol. Immunol.* 32: 101-116, 1995).

A group of membrane proteins known as uroplakins, produced on the apical surface of the bladder urothelium, can form thick protein particles makeing two-dimensional crystals (the "urothelial plaques") that cover over 80% of the apical surface of urothelium (Yu, J. et al.: *J. Cell Biol.* 125: 171-182, 1994; Sun T. T. et al.: *Mol. Bio. Rep.* 23: 3-11, 1996). They are urothelium-specific markers and are conserved during mammalian evolution (Wu, x. R. et al.: *J. Biol. Chem.* 269: 13716-13724, 1994). Recently, using the uroplakin II gene promoter (U.S.P. 5,824,543), transgenic mice that express human growth hormone (hGH) in their bladder epithelium were generated, resulting in the secretion of the recombinant hGH into the urine at 100-500 mg/l (Kerr, D.E. et al.: *Nat. Biotechnol.* 16: 75-79, 1998).

Described below is the construction of the Hu-K20 expression cassettes using a eukaryotic expression vector, pcDNA4/HisMax (Invitrogen). Standard methods are used for plasmid purification, restriction enzyme digestion, DNA litigation, and DNA fragment isolation.

Referring to Fig. 7, construction of the Hu-K20 light chain expression cassette PCR is performed using DNA of the 483 wkmM16 expression vector (Dr. Zhou, Nexia) as the template with a 5' sense primer (5'GCGCAGCAATTGGCGGCCGCTCTAGACTCG3') containing a MunI site

(underlined) and a 3' antisense primer (5'GCGCAGCTCGAGGTCGACGCCCCATCCTCAC3') containing a XhoI site (underlined). The 2.4 kb amplified fragment from upstream of the chicken β-globin gene, used as an insulator, is digested with MunI and XhoI, and ligated at the XhoI site with a 3.6 kb-long XhoI-BamIII fragment of the UPII gene promoter, released from pGI containing the genomic sequence of the UPII (Dr. T. T.Sun, Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York). A pcDNA4/HisMax expression vector (Invitrogen) is digested with MunI and BamHI, and the CMV promoter-less vector is ligated with the MunI-BamHI fragments from pcDNA4/HisMax as the template with a 5' sense primer (5'GCGTATGGATCCAGCGCAGAGGCTTG3') containing a BamHI site and a 3' antisense primer (5'GCGTATTGCATTCGGTTTCGGAGGCCGTCCG3') containing a BswiI site. The amplified SP 163 fragment which serves as a translational enhancer (Gorman, C. M. et al.: *Proc. Natl. Acad. Sci.* 79: 6777-6781, 1982) is digested with BamHI and BswI. A 320 bp fragment of the variable region of the mAb Hu-K20 light chain module is obtained by PCR amplification of pSVhyg-HuVKK20-HuCK plasmid DNA (Paul, M.A. *et al.*, Mol. Immunol. 32: 101-116, 1995) with a 5' sense primer (5'GCGTATTGCATTC*CACC***ATG**GGATGGAGCTGTATCATC3') containing a BswI site (underlined), the Kozak sequence (italics) and the start codon (bold) followed by a partial mouse V47 Ig heavy chain signal sequnce, and a 3' antisense primer (5'GCGTATGTTAACACTTACGTTTGATCTCCAG3') containing a HpI site (underlined). The amplified product is digested with BswiI and HpaI. Another PCR is performed in pSVhyg-HuVKK20-HuCK with a 5' sense primer (5'CGCTATGTTAACGAGTAGACTTAAACACCATCCTGTTTCG3') containing a HpaI site (underlined) a splice donor signal (bold) for the 3' end of

the variable region of the light chain, and partial 5' sequence of the human genomic Ig kappa constant region, and a 3' antisense primer (5'GCGTATGTTTAAACGAGTAGTTGGTAAACAACAG3') containing a PmeI site (underlined) and partial sequence of the human genomic Ig kappa constant region. The PCR product is digested with HpaI and PmeI. The amplified PCR products are ligated together through BswiI and HpaI sites, respectively. The pcDNA4/HisMax/Insulator/UPII is digested with BamHI and PmeI and ligated with the ligated PCR product with overhangs of BamHI site at 5' and PmeI site at 3' to form pcDNA4/Max/UPII-K20L (Fig. 7).

The expression cassette pcDNA4/Mas/UPIIK20L can be used as a versatile system for the cloning and expression of immunoglobulins consisting of heavy and light chains. An advantage of this system is that both the V-genes and the C-genes may be exchanged as cassettes in the vectors, given the low frequency restriction enzyme sites in the Ig genes that have been chosen. In addition, the V-genes can be kept intact, and transient and stable expression of antibodies can be done either from two separate vectors, or from one tandem vector. (Norderhaug, L. et al.: *J. Immunol. Methods* 204: 77-87, 1997).

Referring to Fig. 8, for the construction of the Hu-K20 heavy chain expression cassette, a 360 bp fragment of the variable region of the Hu-K20 heavy chain module is obtained by PCR amplification of pSVgpt-VHK20-HuCyI DNA (Paul, M.A. *et al.*, Mol. Immunol. 32: 101-116, 1995) with a 5' sense primer (5'GCGTATTGCATTC*CACC***ATG**GGATGGAGCTGTATCATC3') containing a BswiI site (underlined), the Kosak sequnce (italic) and the start codon (bold) followed by a partial mouse V47 Ig heavy chain signal sequence , and a 3' antisense primer (5'GCGTATATCGATCTGAGGAGACGGTGACCGTG3') containing a ClaI site (underlined). The amplified product is digested with BswiI and ClaI.

Another PCR is performed in pSVgpt-VHK20-HuCyI with a 5' sense primer (5'CGCTATATCGATAGGTGAGTAGCTTTCTGGGGCAG3') containing a ClaI site (underlined), a splice donor signal (bold) for the 3' end of the variable region of the heavy chaing, and partial 5' sequence of the human genomic Ig gamma-1 constant region, and a 3' antisense primer (5'GCGTATGTTTAAACGACCCGCTCTGCCTCCCTC3') containing a PmeI site (underlined) and partial 3' sequence of the human genomic Ig gamma-1 constant region. The PCR product is digested with ClaI and PmeI. The two amplified PCR products are joined through the ClaI site. The pcDNA4/Max/UPII-K20L vector is digested with BswiI and PmeI and ligated with the ligated PCR product with overhangs of BswiI site and 5' and PmeI site at 3' to form pcDNA4/Max/UPII-K20H (Fig. 9).

A co-expression vector containing both light chain and heavy chain modules is generated as follows. The pcDNA4/Max/UPII-K20H is digested with XhoI and PmeI. The digested XhoI-PmeI fragment is gel-purified, the XhoI sticky end is filled with Klenow, and blunt-end ligated tot he pcDNA4/Max/UpH-K20L already digested with PmeI, to form pcDNA4/Max/UPII-K20LH. The orientation of the pcDNA4/Max/UPII-K20LH is verified by the digestion of the vector with HpaI and ClaI.

A human urothelium cell line (hu609) (Stacey, S.D. et al.: *Mol. Carcinog* 3: 216-225, 1990) is to be used to assess the established expression cassettes. The DNA of the L chain and the H chain constructs is purified by a Maxi-preparation (SOP#008) and is introduced simultaneously at equal molar concentration into the Hu609 cell line by standard transfection techniques. Culture supernatant is precipitated and applied to an anti-human IgG (H chain specific)-Agarose column (Sigma). Protein concentration of eluted fractions is assayed by Bradford microassay (Bio-Rad) and fraction containing proteins are checked on a 10% SDS-PAGE. The expressed proteins are also detected by

Western blot with anti-IgG (H chain) antibody (Sigma).

Transgenic animals expressing this construct are generated as follows. MunI-PmeI digestion of the expression vectors pcDNA4/Max-UPII-K20L, and PCDNA4/Max-UPII-K20H, respectively, releases the L chain and the H chain fragments for microinjection. Hu-K20 transgenic mice are generated either by 1) co-injecting the L chain and the H chain fragments in a 1:1 molar ratio, or 2) injecting the MunI-PmeI fragment of the co-expression vector pcDNA4/Max-UPII-K20LH. DNA is purified and injected by standard techniques. Hu-K20 transgenic goats will be generated by injecting the same transgenes.

Example VII

Expression of the monoclonal antibody K20 under the control of the goat uromodulin gene promoter in a eukaryotic expression vector, pcDNA4/HisMax.

To clone the goat uromodulin gene promoter, PCR was performed using goat genomic DNA as template, with two sets of primers designated from conserved regions of human and cattle UM gene promoters. A 600 bp fragment was obtained and sequenced. It shared 94% and 67% identity with the known sequences of bovine and human UM gene promoters, respectively. Several specific genomic libraries were constructed with the Universal Genome Walker Kit (Clonetech) and PCR was performed using the libraries as templates with several gene-specific primers designated from the 600 bp fragement of the goat UM gene promoter and specific adaptor primers. A 1.5 kb fragment, which includes the 600 bp piece, was obtained both from one of the libraries ad the goat genomic DNA. This 1.5 kb fragment was subcloned into a promoter-less pEGFP to form pGUEGFP3, and sequenced to its entirety (Fig. 10).

In the alternative strategy the genomic uromodulin gene including the

promoter and 3'-elements is cloned by standard techniques and the gene of the interest is fused just before uromodulin's signal peptide or using uromodulin's signal sequences. In addition, the 3' end elements of the uromodulin structural gene including introns can be fused to the 3' end of the gene of the interest.

The goat UM gene promoter was used in the construction of Hu-K20 light chain and heavy chain expression. PCR was performed using as a template the 483 wkmM16 expression vector DNA (courtesty of Dr. Zhou, Nexia Biotechnologies Inc.) that harbors 2 copies of the chicken β-globin insulator sequences in a head to tail orientation (Chung, J. H. et al.: *Proc. Natl. Acad.* 94: 575-580, 1997) with a 5'sense primer (5'GCGCAGCAATTGGCGGCCGCTCTAGACTCG3') containing a MunI site (underlined) and a 3' antisense primer (5'GCGCAGCTCGAGGTCGACGCCCCATCCTCAC3') containing a XhoI site (underlined) (Chung, J. H. et al.: *Proc. Natl. Acad. Sci.* 94: 575-580, 1997). The amplified 2.4 kb insulator fragment of the chicken β-globin gene was digested with MunI and XhoI, and ligated at the XhoI site with a 1.5Kb-long XhoI-BamHI fragment of the goat UM gene promoter, released from PGUEGFP3. The pcDNA4/HisMax expression vector (invitrogen) was digested with MunI and BamHI, and this CMV promoter-less vector was ligated with the MunI-BamHI combined fragment of the insulator and the goat UM promoter. PCR was performed using the excised MunI-BamHI fragment containing the SP163 sequences (Gorman, C. M. et al.: *Proc. Natl. Acad. Sci.* 79: 67777-6781, 1982) from pcDNA4/HisMax as the template with a 5' sense primer (5'GCGTATGGATCCAGCGCAGAGGCTTG3') containing a BamHI site and a 3' antisense primer (5'GCGTATTGCATTCGGTTTCGGAGGCCGTCCG3') containing a BswiI site. The PCR product was digest with BamHI and BswiI. The amplified SP163 fratment is derived from the 5' untranslated region (UTR) of the vascular

endothelial growth factor (VEGF) gene and it has been shown to increase expression levels two-to-five fold above those seen with promoter along (gorman, C. M. et al.: *Proc. Natl. Acad. Sci.* 79: 6777-6781, 1982). A 320 bp fragment of the variable region of the mAb Hu-K20 light chain module was obtained by PCR amplification of pSVhyg-HuVKK20-HuCK plasmid DNA (Paul, M.A. *et al.*: *Mol. Immunol.* 32: 101-116, 1995) with a 5' sense primer (5'GCGTATTGCATTC*CACC***ATG**GGATGGAGCTGTATCATC3') containing a BswI site (underlined, the Kozak sequence (italic) and the start codon (bold) followed by a partial mouse V47 Ig heavy chain signal sequence, and a 3' antisense primer (5'GCGTATGTTAACACTTACGTTTGATCTCCAG3') containing a HpaI site (underlined). The amplified product was digested with BswiI and HpaI. Another PCR was performed in pSVhyg-HuVKK20-HuCKL with a 5'sense primer (5'CGCTATGTTAAC**GAGTAGA**CTTAAACACCATCCTGTTTCG3') containing a HpaI site (underlined), a splice donor signal (bold) for the 3' end of the variable region of the light chain, and partial 5' sequence of the human genomic Ig kappa constant region, and a 3' antisense primer (5'GCGTATGTTTAAACGAGTAGTTGGTAAACAACAG3') containing a PmeI site (underlined) and partial 3' sequences of the human genomic Ig kappa constant region. The PCR product was digested with HpaI and PmeI. The three amplified PCR products were ligated together through BswI and HpaI sites with the resulting fragment having BamHI and PmeI ends. This joined PCR product with overhangs of BamHi site at 5' and PmeI site at 3' was ligated to pcDNA4/HisMa/Insulator-UM which was digested with BamHI and PmeI, yielding pcDNA4/Max/UM-K20L (Fig. 8).

For the construction of the Hu-K20 heavy chain expression cassette a 360 bp fragment of the variable region of the Hu-K20 heavy chaing module can

be obtained by PCR amplification of PSVgpt-VHK20-HuCγ1 DNA (Paul, M.A. *et al.*: *Mol. Immunol.* 32: 101-116, 1995) with a 5' sense primer (5' GCGTATTGCATTC*CACC***ATG**GGATGGAGCTGTATCATC3') containing a BswI site (underlined), the Kozak sequence (italic) and the start codon (bold) followed by a partial mouse V47 Ig heavy chain signal sequence, and a 3' antisense primer (5'GCGTATATCGATCTGAGGAGACGGTGACCGTG3') containing a ClaI site (underlined). The amplified product is digested with BswiI and ClaI. Another PCR is peformed in pSVgpt-VHK20-HuCγI with a 5' sense primer (5'CGCTATATCGAT**AGGTGAG**TAGCTTTCTGGGGCAG3') containing a ClaI site (underlined), a splice donor signal (bold) for the 3' end of the variable region of the heavy chain, and partial 5' sequence of the human genomic Ig gamma-1 constant region, and a 3' antisense primer (5'GCGTATGTTTAAACGACCCGCTCTGCCTCCCTC3') containing a PmeI site (underlined) and partial 3' sequence of the human genomic Ig gamma constant region. The PCR product is digested with ClaI and PmeI. The two amplified PCR products are joined through the ClaI site. This joined PCR product with overhangs of BswI site at 5' and PmeI site at 3' is ligated to pcDNA4/Max/UM-K20L which is digested with BswiI and PmeI to form PCDNA4/Max/UM-K20H (Fig. 8).

A single vector containing both light chain and heavy chain modules for co-expression can be generated by inserting the XhoI-PmeI digested and 5' end blunt-ended UM promoter plus H chain module of pcDNA4/Max/Um-K20H into the PmeI-digested pcDNa4/Max/UM-K20L to form pcDNA4/Max/UM-K20LH (see Fig. 9). Alternatively, a bicistronic expression vector can be constructed. In this case a single goat UM promoter is driving expression of both the light and heavy chains. For this purpose, the second UM promoter fragment in pcDNA4/Max/UM-K20LH can be replaced by a fragment of an internal ribosomal entry site (IRES) sequence (Staal, F.J. et al.: Cancer Gene

Ther 3: 345-351, 1996).

Testing of the constructs in renal epithelial cell lines

Prior to the generation of transgenic animals using the transgenic constructs, their functionality can be determined upon transfection in kidney epithelial cells and media testing for the presence of the antibody. For example, the rabbit renal cell line, PAP-HT25 (Green, N. et al: Am J Physiol 249:C97-104. 1985), and the dog kidney cell line MDCK (ATCC #CCL-34) can be used to test the functionality of the established expression cassettes. The cells can be transfected with the L chain and H chain constructs simultaneously at equal molar concentration by standard transfection technique. The co-expression vector can also be transfected into the same cell lines. Twenty-four hours following transfection, the cells conditioned media can be exchanged with fresh media, and the cells returned to culture. The next day, the conditioned media of the cells can be removed and applied on an anti-human IgG (H chain specific)-Agarose column (Sigma) for antibody purification or tested directly for the presence of the antibody by Western blotting analysis. Protein concentration of eluted fractions is assayed by Bradford microassay (Bio-Rad) and fractions containing proteins can be checked on a 10% SDS-PAGE. Other functional assays for the recombinant mAb Hu-K20, for example, binding measurement, T cell proliferation, measurement of phosphatidic acid synthesis, complement-dependent cytotoxicity assay and measurement of C1q binding can also be performed.

Generation of the transgenic animals with the Hu-K20 transgenes under the control of the goat UM gene promoter

Generation of transgenic animals can be performed using standard techniques including pronuclear microinjection (Wright, G. et al.: Bio/Technology 9:830-83, 1991; Pursel, V.G. et al.: J Anim Sci 71 Suppl 3:1-9, 1993; Wall, R.J. et al.:

Theriogenology 5:57-968, 1996) or nuclear transfer (NT) methodologies (Campbell, K.H. et al.: Nature 380:64-66, 1996; Wilmut, I. et al.: Nature 385:810-813, 1997; Cibelli, J.B. et al.: Science 280: 1256-1258, 1998; Wakayama, T. et al.: Nature 394: 369-374, 1998). MunI-PmeI digestion of the expression vectors pcDNA4/Max/UM-K20L and pcDNA4/Max/UM-K20H, respectively, removes the vector backbone and generates the insulator, UM promoter and the L chain and the H chain fragments for microinjection. Transgenic animals can also be generated by co-injecting the L chain and the H chain fragments in a 1:1 molar ratio. The transgenic animals can also be generated by injection of the MunI-PmeI fragment of the co-expression vector, pcDNA4/Max/UM-K20LH. DNA is purified and injected by standard techniques. Genomic DNA of the putative transgenic animals is prepared and analyzed by PCR and Southern blot. NT derived offspring cell lines such as fetal fibroblasts can be transfected *in vitro* (Cibelli, J.B. et al.: Science 280: 1256-1258, 1998) with the UM-K20H and UM-K20L expression cassettes selected using for example Zeocin selection marker. Lines are screened for copy number and integration of both cassettes prior to using them in NT experiments. Reconstructed NT embryos can be cultured or transferred immediately to further recipient animals.

Urine of transgenic animals can be collected daily starting at birth and assayed to determine the quantity as well as the quality of the secreted monoclonal antibodies.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further

modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed:

1. A nucleic acid molecule comprising (i) a sequence encoding a polypeptide, (ii) a promoter from a kidney-specific gene, said promoter operably linked to said sequence, and (iii) a leader sequence that enables secretion of said polypeptide by said urine-producing cells into urine of an animal.

2. The nucleic acid molecule of claim 1, wherein the promoter from the kidney-specific gene is selected from the group consisting of a goat, sheep, pig, cow, human, and rodent.

3. The nucleic acid molecule of claim 1, wherein said kidney specific gene is a uromodulin gene.

4. The nucleic acid molecule of claim 1, wherein said animal is a mammal.

5. The nucleic acid molecule of claim 1, wherein said polypeptide has biological activity.

6. The nucleic acid molecule of claim 1, wherein said polypeptide is soluble.

7. An animal in which the genome of cells that contribute to urine production in said animal comprises the nucleic acid molecule of claim 1.

8. The animal of claim 6, wherein said cells are kidney secretory cells.

9. The animal of claim 7, wherein said animal is selected from the

group consisting of a rodent, a ruminant, human or pig.

10. The animal of claim 7, wherein said animal is a mammal.

11. A method for producing a polypeptide, said method comprising the steps of:

(a) providing a cell of an animal, said cell transfected with a nucleic acid molecule that contains (i) a nucleic acid sequence encoding a polypeptide, (ii) a kidney specific gene promoter that directs expression of said polypeptide in said cell, and (iii) a leader sequence that causes secretion of said polypeptide by said cell;

(b) culturing said transfected cell; and

(c) isolating said polypeptide from the culture medium of said cultured transfected cell.

12. The method of claim 11, wherein said promoter is a uromodulin gene promoter.

13. The method of claim 12, wherein said cell is a kidney secretory cell.

14. The method of claim 11 or 12, wherein said animal is a mammal.

15. The method of claim 11 or 12, wherein said kidney specific gene promoter is a uromodulin gene promoter.

16. A method for producing, in the urine of a vertebrate, a recombinant protein that contains two or more subunits linked to each other by disulfide bonds, said method comprising the steps of:

(a) providing a transgenic vertebrate exhibiting urine-specific production
of the recombinant protein;

(b) collecting urine from said vertebrate; and

(c) isolating said protein from said urine.

17. The method of claim 16 wherein said recombinant protein is an antibody.

18. The method of claim 16 wherein said protein is a heterodimeric fertility hormone.

19. The method of claim 16 wherein said protein is collagen or fibrinogen.

20. A transgenic non-human vertebrate animal that exhibits urine-specific production of a recombinant protein that contains two or more subunits linked to each other by disulfide bonds, said protein being folded such that it is rendered biologically active.

21. The animal of claim 20, wherein the DNA encoding said protein is operatively linked to a uromodulin or uroplakin promoter.

22. The animal of claim 21, wherein the DNA encoding said protein is operatively linked to a leader sequence that enables secretion of the protein into the urine of said animal.

23. A purified nucleic acid molecule comprising a goat uromodulin

gene promoter.

24. The nucleic acid molecule of claim 4, wherein said mammal is a goat.

25. The method of claim 16, wherein said protein is folded such that it is rendered biologically active.

26. The method of claim 16, wherein said protein is excreted into the urine of said animal in inactivated or partially inactivated form, said protein being activatable.

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1 cagagtgggt caggtccagt gatgtctgaa ctaccttctg gttctgactt
tcagccattc
61 tcagctcctc tcttgcttgt gtctggattc taaggctgat ctcatgagaa
tgggtgtttc
121 agaagggtgc cctctccaag acaggtgcac ctcccatctg gggcagtgaa
tatccttttg
181 tccttatgca gcctggcttc agatactggc ttctgcctgg ctccttgatc
ccaccctgcc
241 cttgtcagtg accaagaaga agcccagcac cttggcactg ctttcccagt
taatttctaa
301 ctatggaatc tcttgctgtt agaaggtgcg aaacagtgac cttgtatttc
cgggcacagg
361 tgtgaccccc caatgtcaat catttggggt ctctagctat taggaaaaag
aacaacaaca
421 acctcacagc ttggacaagg caaacattat gccaggagga aaaaatattc
caccccaag
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agcctggggc
541 aaatgtgtat atataagtat gaggcacatc atcaccagac taactctacc
tttctggctt
601 caggtaaggc tatctgtagc
//
```

Fig. 1

```
1 aatttcttga ttcacagagc atctggtcca atgatgtctg aattgccttc
tgtctctgac
61 cttcaggcat tctcagctcc tttcctgctc acatcgggac cccagggaag
ctggttgaac
121 ccatgaggat ggaacttgct ttggaactga gtggccacaa gtatacatcc
cagtggggac
181 agtgagcacc ccttttctcc tggagcagcc tggcttcaga ttctggcctc
tgctttccac
241 tttgtgcttt tcaatgacca agaaaagccc aggcacttgg aatttttttac
ccagttaatt
301 tctaactaaa gaacctctcg ttgccaaaag atataaaaca gagcccttgt
aactctgggc
361 acaactgtga ccccagtgtc aatcatttgg ggtctctacc tattagggaa
aagaacaaca
421 accacctcac agcctagaaa aggaaaacac tgtgtcaaaa gggaaaaata
ttccaccccc
481 attaaaataa ttaagaaaca gaaccagagg atcattggag gaaagactgc
cagtgggggа
541 cagatgtata tatatagata tgatagtcac ctacttgtaa aaggattaat
tctacctttc
601 tggtttcagg taaggctatc tgcaga
```

Fig. 2

```
1 ctagtcttgt ctgacagagg tccagttgag ggatgtccag atggtcttgc
aaccgataac
61 tttctcagag actctctctt tcctgtctgg actctagtgg ggaggactaa
tctggtgaag
121 ctgttcttca gatcaggtgt gtgttccagg cttcgaagca aatgtttctg
ttatcctaac
181 ccaggctggc ttcagatatt gtctttttc ctgccccttt ggtatttcca
ccttgtcctt
241 gcccaggtcc aagaaaaagc ccagaacctt ggcactgctt tgccagttaa
tgtctaaccg
301 aggaatgtct tgctgccaaa aggtgcaaac agagaccttg tatttccagg
cacaggtgtg
361 acccccaatg tcaatcattt tctgtctcta actaccagag gaaaaactaa
caacaacagc
421 ctcatggttt ggaaaaggtg aactctatgc caaatgggaa gaaaaattct
gacccccaca
481 gaaacaatct caagaggcag aagcagagaa taattggagg gagagggcca
gccaagggca
541 gacatatata tatatatatt gatcacaggc acttacttgt gaatggacca
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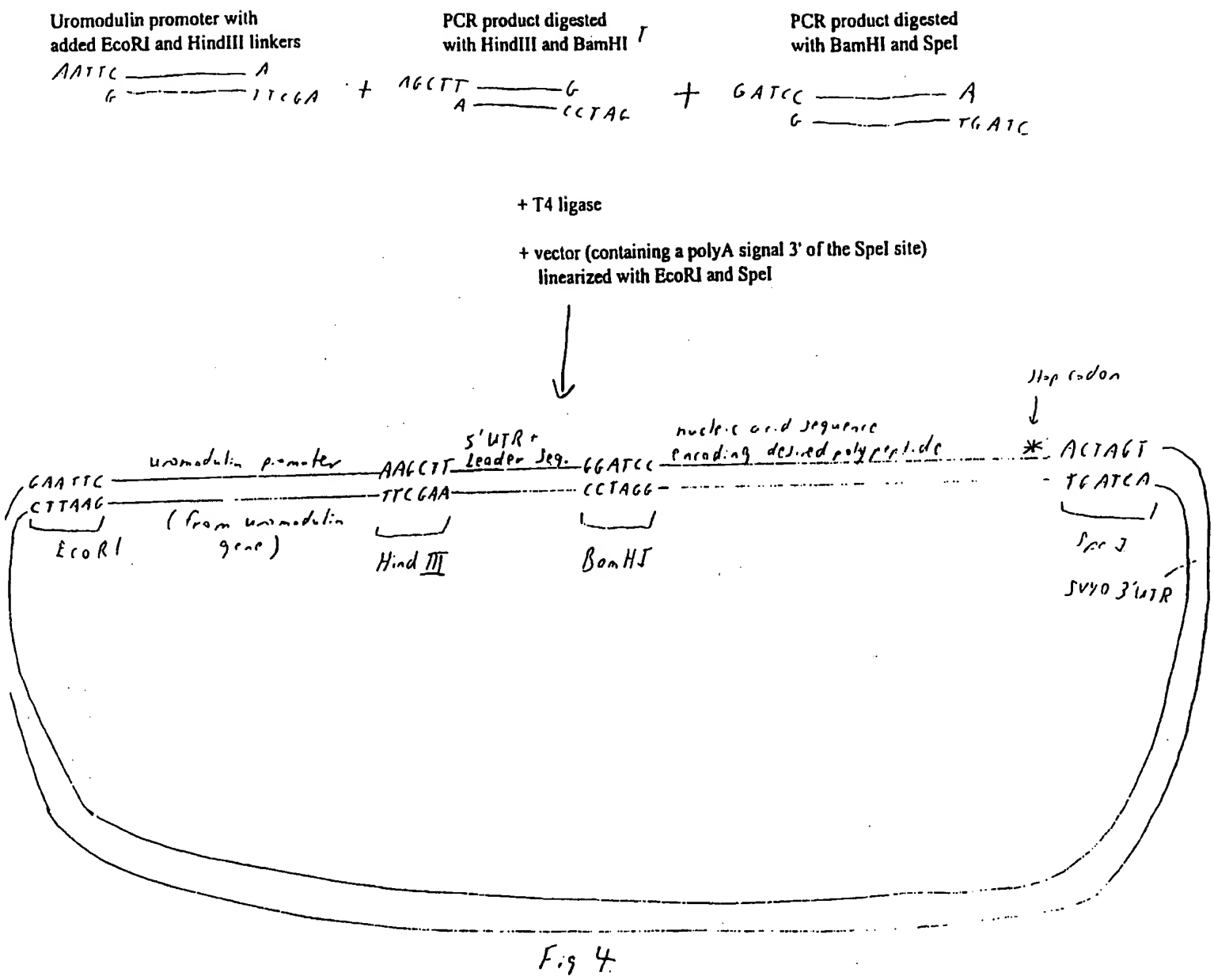
Fig. 3


Uromodulin promoter with added EcoRI and HindIII linkers
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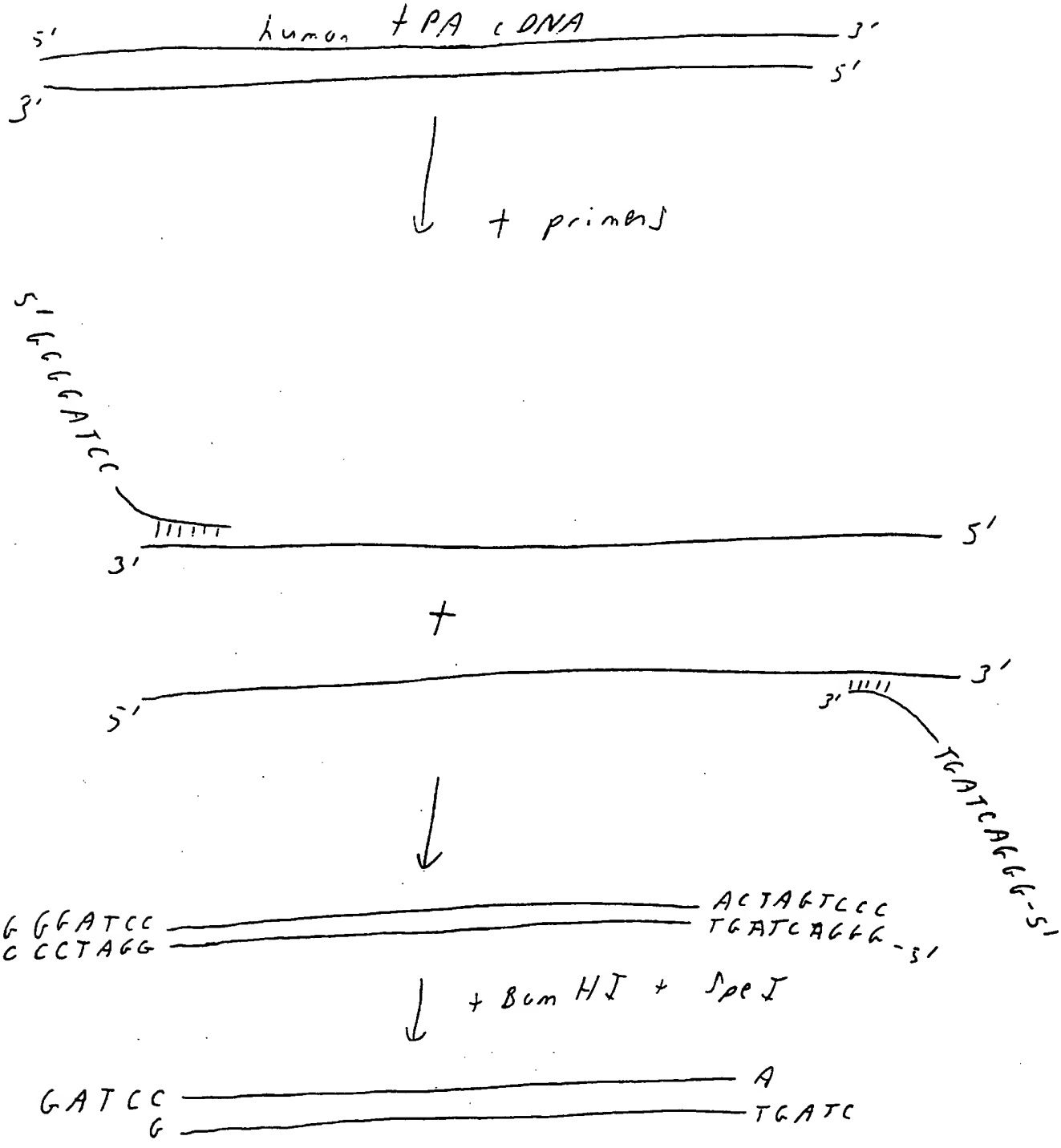
G
TTCGA
+
PCR product digested with HindIII and BamHI
AGCTT
G
A
CCTAG
+
PCR product digested with BamHI and SpeI
GATCC
A
G
TGATC
+ T4 ligase
+ vector (containing a polyA signal 3' of the SpeI site) linearized with EcoRI and SpeI
Stop codon
5'UTR + Leader Seq.
nucleic acid sequence encoding desired polypeptide
uromodulin promoter
GAATTC
CTTAAG
AAGCTT
TTCGAA
GGATCC
CCTAGG
*
ACTAGT
TGATCA
(from uromodulin gene)
EcoRI
Hind III
BamHI
Spe I
SV40 3'UTR

Fig 4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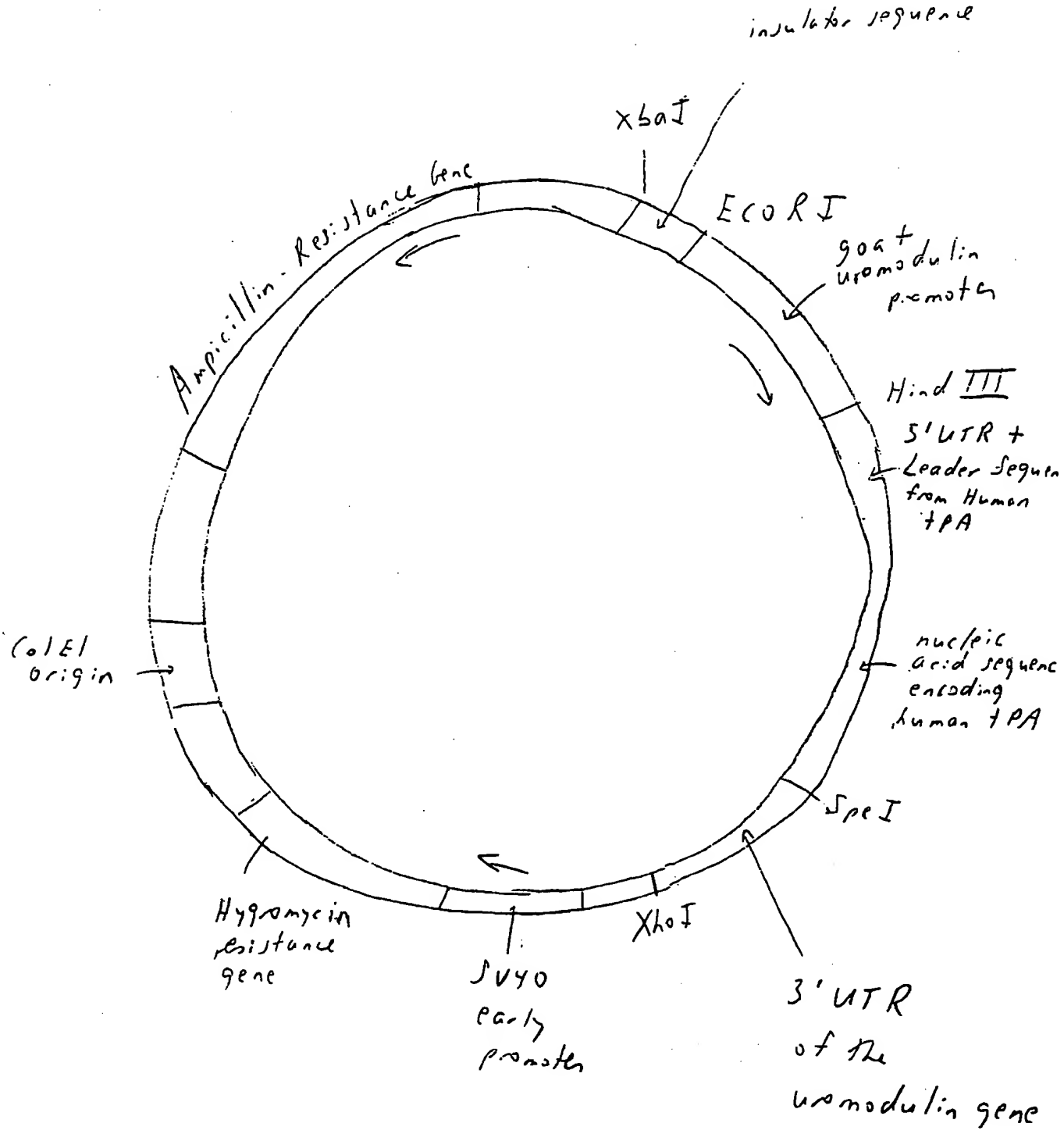
human t PA cDNA
3'
5'
3'
+ primers
5'
GGGGATCC
3'
5'
+
3'
5'
3'
TGATCAGGG-5'
ACTAGTCCC
GGGGATCC
TGATCAGGG-5'
CCCCTAGG
+ Bam HI + Spe I
A
GATCC
TGATC
G

Fig. 5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EcoRI
goat uromodulin promoter
HindIII
5'UTR + Leader Sequen from Human tPA
nucleic acid sequenc encoding human tPA
SpeI
XhoI
3'UTR of the uromodulin gene
SV40 early promoter
Hygromycin resistance gene
ColEI origin
Ampicillin-Resistance Gene

Fig. 6

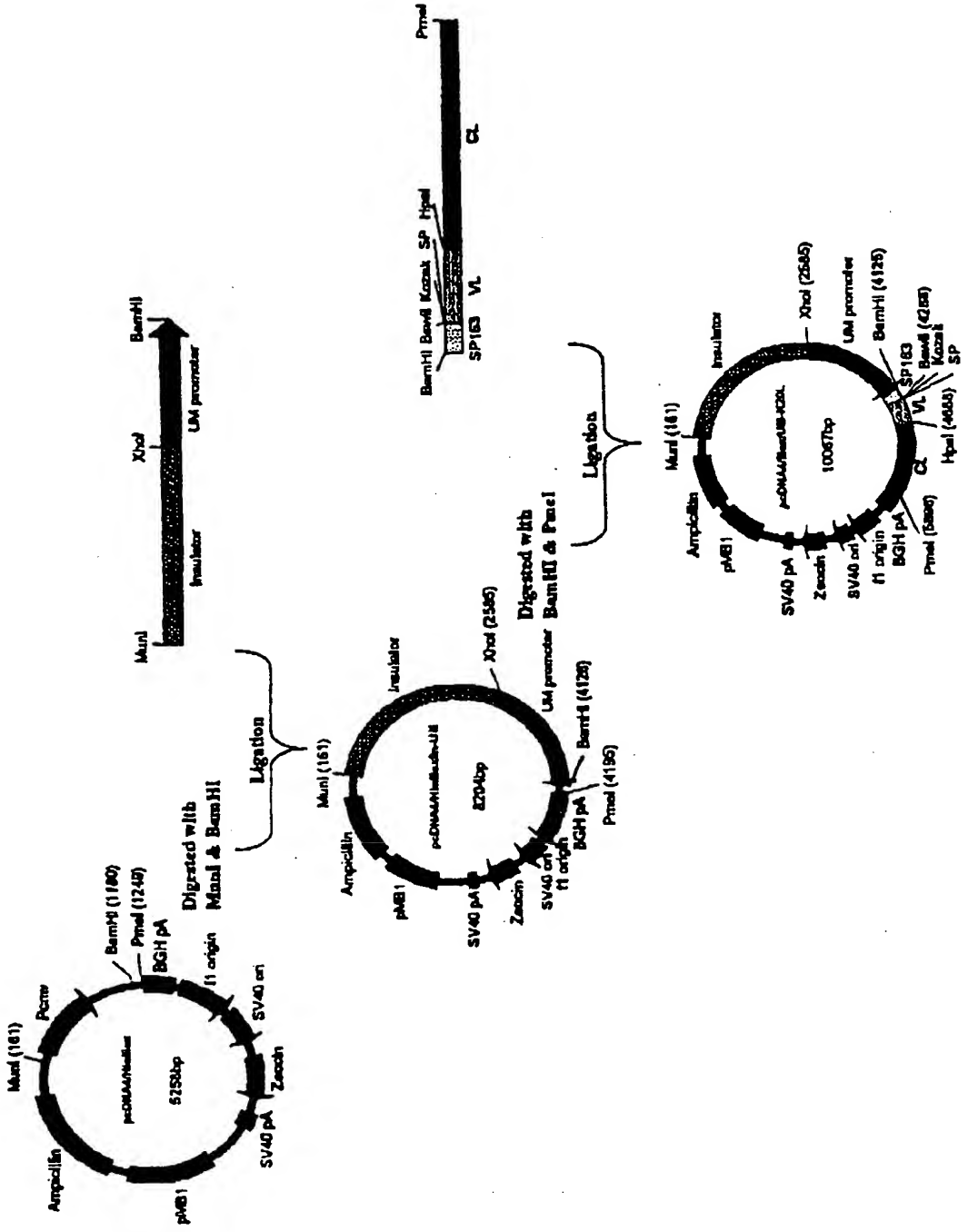


Fig. 7 Construction of expression vector pcDNA4/Max/UM-K20L. SP, sequence for signal peptide; VL, light chain variable region; CL, light chain constant region.

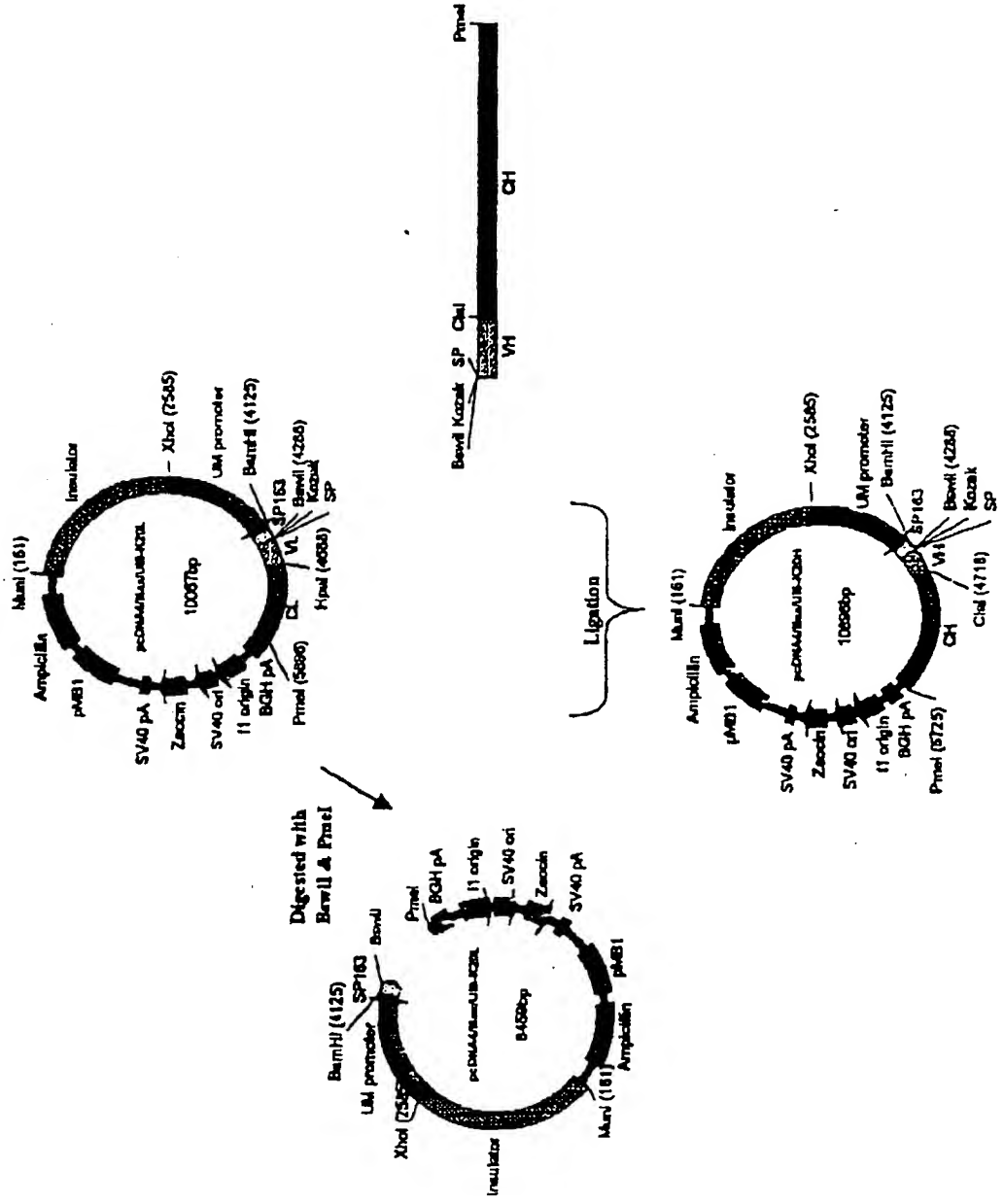


Fig.8 . Construction of expression vector pcDNA4/Max/UM-K20H. SP, sequence for signal peptide; VH, heavy chain variable region; CH, heavy chain constant region.

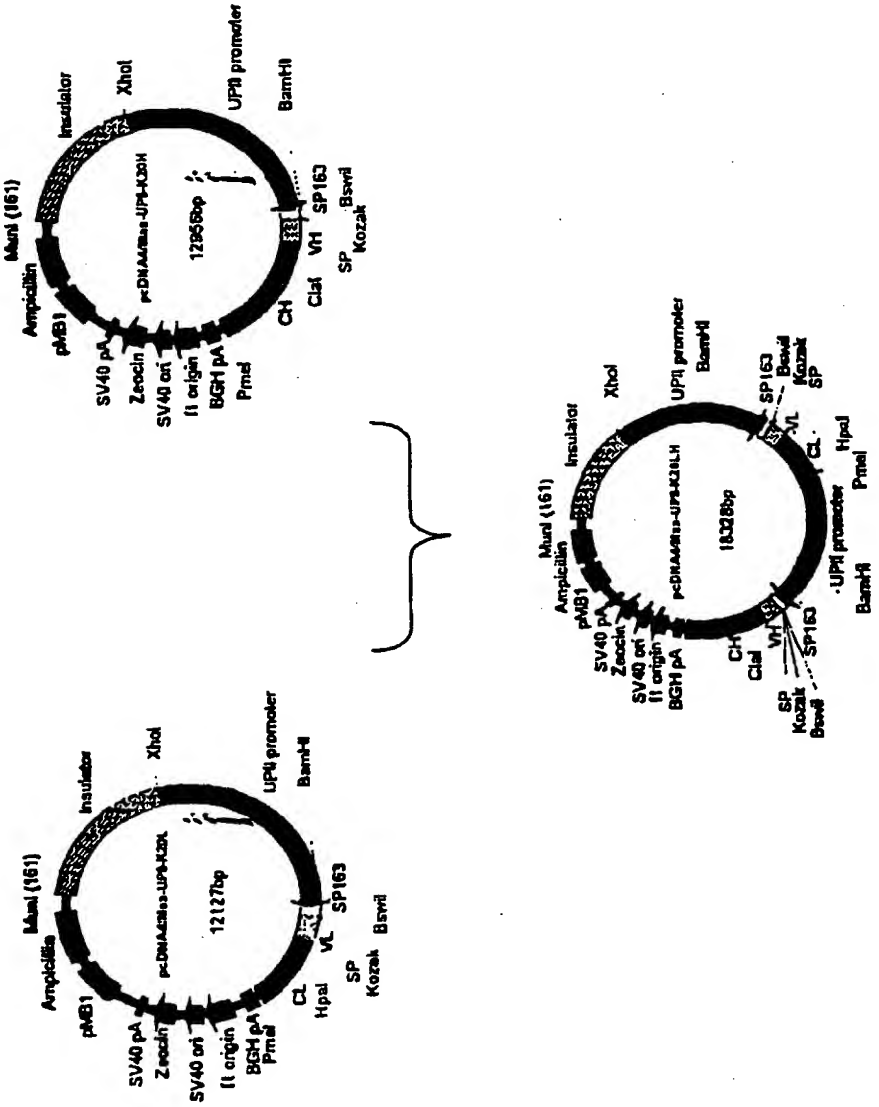
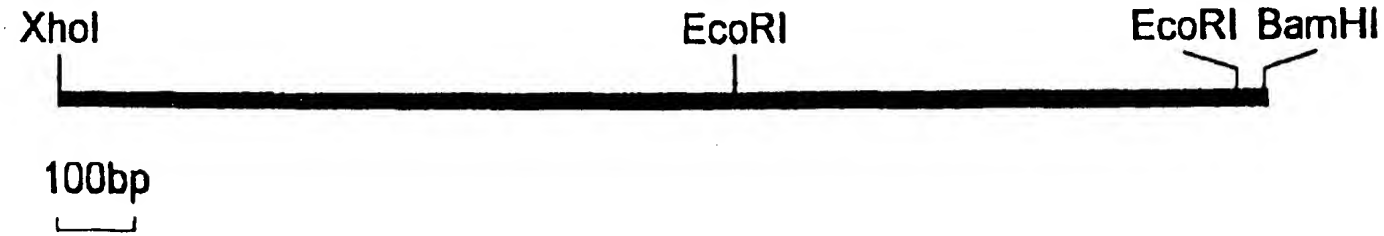


Fig. 9 Construction of expression vector pcDNA4/Max-UPII-K20LH. SP, sequence for signal peptide; VL, light chain variable region; CL, light chain constant region; VH, heavy chain variable region; CH, heavy chain constant region.

A



B

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-1258  TTTTTCATCA AAGAACTGCA AAAGTAAATA ATGATAAGAT GTTTCTCACT TTTCCACAAA GATGAAAGTT AATGCCCAGG GTGGCTGAGT ACTGTGCTGG
-1158  GATTGTGAAC TAACTGTTAT AGATCTCTCT GGGGTGCTGT TTGGGAAGAA ACATCGCTGA AAACTGAGCT ACCTCTTTTC CTATGAAATT CCCCTGAGGA
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 -958  TCAGCCTGAG TTCACATGTG CCTGATTTTT CTCAATATGG CATTGCCATT AACGTGGAAT TAGGTCAGGA GACCTAAGGC TGAACCAAGC CCTGTCATTC
 -858  TCTGCCCCAT GACTGCGCAT CACCAAAACA GCATCGGCAG TGACTTCCAC AGATGGTACC ATTGCTATAT GCCTTAACTT GCATCATCTC CTTTAATGGC
 -758  CATAACAATT CTAGGACACG GGTATTCTTG TTTTACAGAT GATGAAAATT ACCTCTGGAA GGAAAATTAC TGGCACACAA AAAACGCTGA CCAGGATTCA
 -658  GATAGACTGA CTCCAAAGTC AGTCTGTTCA TCTACAAAAT TATCTACTTC TCAAGGACCT TCCTTCATGG GAATTCAAAT TTCTTGATTC ACAGAGCATC
 -558  TGGTCCAATG ATGTCTGAAT TATCTGCTGT CTCTGACCTT CAGCCATTCT CAGCTCCTTT CCTGATCACA TTGGGACCCC AGGGGAGCTG GCTGAATCTG
 -458  TGAGGATGAC ATTTGCTTTG GAATTAAGTG GCCACAAGTA CACATCCTGG TGGGGACAAT GAGCACCCCT TTTCTCCTGG AGCAGCCTGG CTTCAGATTC
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 -258  TTGCCAAAAG GTATAAAACA GAGCCCTTGT AACTGTGGGC ACAGCTGTGA CCCCCATGTC AATCATTTGG GGTCTCTACC TATTAGGGAA AAGAACAACA
 -158  ACCACCTCAC AGCCTAGAAA AGGAAAACAC TGTGTCAAAA GGGAAAAATA TTCCACCCCC ATTAAAATAA TTAAGAAACA GAACCAGAGG ATCATTGGAG
  -58  GAGAGATTGC CAGTGGGGGA CAGATGTATA TATATAGATA TGAAAGTCAC CTACTTGTAA AAGGATTAAT TCTACCTTTC TGGTTTCAGG AAGGTACCAC
   52  GTAGCCGAAT TCTGCAGTCG ACGGACGGTA CCGCGGGCCC GGGATCC
```

Fig. 10 Sequence of the goat UM gene promoter. A: a partial restriction map; B: nucleotide sequence of the 1.5 kb fragment of the promoter. The arrow indicates putative transcription initiation site. TATA and inverted CCAAT boxes are underlined according to Yu, H. et al: Gene Expression 4: 63-75, 1994.

SEQUENCE LISTING

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USING A KIDNEY-SPECIFIC GENE PROMOTER

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